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Interactions of Various Air Pollutants on Causation of Pulmonary Disease

IIT Research Institute

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(Final Report)

INTERACTIONS OF VARIOUS AIR POLLUTANTS
ON CAUSATION OF PULMONARY DISEASE

Environmental Protection Agency
Research Triangle Park
North Carolina 27711

Attention: Dr. David Coffin

Report No. IITRI-L6061-8
(Final Report)

INTERACTIONS OF VARIOUS AIR POLLUTANTS
ON CAUSATION OF PULMONARY DISEASE

September 28, 1970 to September 27, 1972

Contract No. EHSD 71-37
IITRI Project L6061

Prepared by
Richard Ehrlich
and
James D. Fenters

of
IIT RESEARCH INSTITUTE
10 West 35th Street
Chicago, Illinois 60616

for
Environmental Protection Agency
Research Triangle Park
North Carolina, 27711
Attention: Dr. David Coffin

Copy No. _____

October 30, 1972

IIT RESEARCH INSTITUTE

FOREWORD

This is Report No. IITRI L6061-8 (Final Report), entitled "Interactions of Various Air Pollutants on Causation of Pulmonary Disease," Contract No. EHSD 71-37, IITRI Project No. L6061. The studies were conducted by IIT Research Institute for the Environmental Protection Agency during the period from September 28, 1970 to September 27, 1972. The report summarizes various studies on the effect of exposure to nitrogen dioxide, chromium trioxide, and nickel oxide on the resistance of experimental animals to bacterial and viral infection. A chronic exposure study to determine the effects of nitrogen dioxide on the immune system initiated during the program will continue beyond September 27, 1972. However, all data available to date are summarized in this report period.

In conducting the research reported, the investigators adhered to the "Principles of Laboratory Animal Care" as established by the National Society for Medical Research.

Dr. Richard Ehrlich served as the principal investigator and Dr. James Fenters as the co-investigator. Other personnel participating in the program were Mr. J. C. Findlay, serving as the principal professional assistant, Mrs. Vivian Neary, Mr. T. Sharp, Mr. W. Jeter, and Mrs. A. Garner. Dr. Curtis D. Port was responsible for all histopathologic examinations, and in conjunction with Miss Catherine Aranyi, performed the scanning electron microscopic examinations.

The experimental data are recorded in IITRI Logbooks
C20305, C20307, C20416, C20495, C20604, D1756, C20892, C20951,
and C21056.

Respectfully submitted,
IIT RESEARCH INSTITUTE

James D. Fenters
James D. Fenters
Senior Microbiologist
Life Sciences Research

Approved by:

Richard Ehrlich
Director
Life Sciences Research

JDF/kk

IIT RESEARCH INSTITUTE

ABSTRACT

Squirrel monkeys were challenged periodically with five intratracheal injections of monkey adapted influenza A/PR/8 virus while continuously exposed for 493 days to ≤ 1 ppm of nitrogen dioxide (NO_2) or filtered air. Exposure to NO_2 appeared to influence the serum neutralization (SN) antibody formation but hemagglutination-inhibition (HI) antibody titers were similar in control and experimental groups of monkeys. Monkeys exposed to NO_2 produced SN antibody within 21 days after virus infection, and after 294 days they consistently showed higher SN antibody titers than the controls. After administration of an inactivated A₂/Taiwan vaccine, the control and experimental monkeys exhibited similar immunogenic responses to both the A₂/Taiwan and A/PR/8 influenza viruses. Throughout the exposure, no significant differences were observed in body temperature, respiratory function, body weight, and hematological values between control and experimental monkeys. Histopathological examination of lung tissue indicated slight emphysema and thickened bronchial epithelium only in monkeys exposed to NO_2 and challenged with influenza virus. Scanning electron microscopic examination also suggested presence of emphysema in a monkey exposed to NO_2 and influenza virus.

Enhanced mortality was observed among hamsters infected with K. pneumoniae within 1 hr after intratracheal instillation of 5 and 7.5 mg of nickel oxide (NiO). Similarly, increased mortalities were observed in hamsters challenged with influenza virus and exposed to NiO. However, the statistical significance of this observation could be demonstrated only for hamsters exposed to 5 mg of NiO 48 hr after the infectious challenge.

Histopathologic examination of lung tissues indicated a more severe pathologic response in hamsters challenged with the virus and given NiO than in the various control hamsters. Clearance studies of influenza virus from lungs indicated little difference in virus titers between experimental and control groups of hamsters.

Single or multiple exposures to chromium trioxide (Cr_2O_3) aerosol did not enhance mortality in mice challenged with K. pneumoniae or A/PR/8 influenza virus. Furthermore, no significant differences in mortalities were observed among infected mice exposed to a combination of Cr_2O_3 aerosol and NO_2 .

A study was initiated to determine the effects of continuous, long-term exposure to NO_2 on the immune response in mice. Mice were exposed to either filtered air, 2 ppm of NO_2 , or 0.5 ppm of NO_2 with daily 1 hr peaks of 2 ppm NO_2 for 5 days a week for 3 months prior to vaccination with an influenza vaccine. After the vaccination, exposure to the various environments was continued. The initial SN antibody response was depressed in mice exposed continuously to 0.5 ppm NO_2 and those held in filtered air prior to vaccination and exposed to 2 ppm or 0.5 ppm NO_2 after vaccination. Within 2 weeks after vaccination the SN seroconversion rate was 100% in the filtered air control mice, whereas no mice in the group exposed to 0.5 ppm of NO_2 seroconverted. Furthermore, the seroconversion rate for the remaining experimental groups of mice ranged from only 40 to 57%. After challenge with live A₂/Taiwan influenza virus, mortality rates and lung lesion scores indicated protection in all vaccinated mice. However, more severe lung lesions

were seen in vaccinated mice or control mice given saline and held in NO₂ for 3 months prior to vaccination than in corresponding groups of mice maintained in filtered air. Scanning electron microscopic examination of lung tissues indicated changes attributable to NO₂ in mice exposed to 2 ppm NO₂ for 16 weeks. Macrophage cells from mice exposed to either 2 or 0.5 ppm of NO₂ for 21 weeks showed distinct morphologic alterations. In some instances, a complete deterioration of the cells, with only some skeletal remnants remaining, was seen.

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A. LONG-TERM EXPOSURE OF SQUIRREL MONKEYS TO NO₂

The studies designed to determine the effects of long-term exposures to nitrogen dioxide (NO₂) in squirrel monkeys were described in detail in Report No. IITRI L6061-4 (Annual Report) dated September 27, 1971. The following is a brief summary of the results.

Squirrel monkeys were challenged periodically with five intratracheal injections of monkey adapted influenza A/PR/8 virus while exposed 24 hr/day, 7 days/week, for 493 days to ≤ 1 ppm of NO₂ or filtered air. Exposure to NO₂ appeared to influence the serum neutralization (SN) antibody formation. All five monkeys exposed to NO₂ produced SN antibody within 21 days after virus infection, whereas only one control monkey showed comparable response. After 294 days, the monkeys exposed to NO₂ consistently showed higher SN antibody titers than the controls. The hemagglutination-inhibition (HI) antibody titers in the control and experimental groups of monkeys did not differ significantly. After administration of an inactivated A₂/Taiwan influenza vaccine, the control and experimental monkeys exhibited similar immunogenic responses to both the A₂/Taiwan and A/PR/8 influenza viruses.

During the 493 day exposure no significant differences were observed in body temperature, respiratory function, body weight, and hematological values between control and experimental monkeys. Histopathological examination of lung tissue indicated slight emphysema and thickened bronchial and bronchiolar epithelium only in monkeys exposed to NO₂ and challenged with the influenza virus. Scanning electron microscopic examination also suggested the presence of emphysema in lungs of one monkey exposed to NO₂ and challenged with the virus. Transmission electron microscopic examination did not show any ultrastructural changes which could be attributed to the experimental exposures.

Increase in chromosome or chromatid breaks was not observed. However, there was an unusually high number of tetraploid metaphases in three of four monkeys exposed to NO₂ and in only one of three controls.

The two publications resulting from work conducted during this phase of the program were:

Antibody response in squirrel monkeys given influenza virus and continually exposed to nitrogen dioxide.
J. D. Fenters, J. Findlay, and R. Ehrlich. Bacteriol. Proceed., 1972.

Immunologic, physiologic, and pathologic effects of chronic exposure to nitrogen dioxide in virus-challenged squirrel monkeys. J. D. Fenters, J. Findlay, C. D. Port, R. Ehrlich, and D. L. Coffin. Submitted for publication in Arch. Environ. Health.

B. INTERACTION OF NICKEL OXIDE AND RESPIRATORY INFECTION
IN HAMSTERS

I. Introduction

This study was conducted to determine the effects of various concentrations of nickel oxide (NiO) on the resistance of hamsters to influenza infection and bacterial pneumonia. The parameters of interest were mortality rates, lung histopathology, and clearance rates of virus from the lungs.

II. Materials and Methods

Experimental Animals. Male Syrian golden hamsters, 6 to 7 weeks old, were used. Throughout the experiments food and water were provided *ad libitum*.

Infectious Agents. Influenza virus A/PR/8 strain was obtained from Dr. Max Rosenbaum, Naval Medical Research Unit No. 4, North Chicago, Illinois. This virus was passaged several times in hamsters and a 20% lung suspension was used for the infectious challenge. Prior to use in the studies, the virus was identified by use of specific A/PR/8 influenza virus anti-serum obtained from the National Institutes of Health. Bacterial challenge was made with mouse adapted Klebsiella pneumoniae type A, strain A-D.

Administration of NiO. Hamsters were anesthetized with dry ice (CO₂) or sodium methohexital and injected intratracheally with sterile 0.2 ml doses containing various concentrations of NiO. The NiO particles suspended in 0.5% gelatin-saline were $\leq 5\mu$ in diameter.

Histopathology. Upon autopsy lungs and hearts of the hamsters were removed as a unit, and representative tissues were fixed in a 10% phosphate buffered formalin solution. After blocking in paraffin, 4 μ thick sections were cut in a rotary microtome and stained with hematoxylin and eosin.

Experimental Protocol. For challenge with K. pneumoniae anesthetized hamsters were injected intratracheally with sterile 0.2 ml of gelatin-saline containing 1, 5, 7.5, and 10 mg of NiO particles. At 1, 6, and 24 hr after the NiO injections, the hamsters were challenged with the bacterial aerosol.

For influenza virus studies, anesthetized hamsters were injected intratracheally with 0.2 ml doses of gelatin-saline containing 1, 2.5, and 7.5 mg of NiO particles. In one group of experiments the hamsters were challenged by intranasal instillation with influenza virus at 1, 24, and 48 hr before the NiO injection. In another group of experiments the effect of a reverse sequence of exposures, namely intratracheal injection of NiO followed by virus challenge, was investigated.

III. Results and Discussion

1. Klebsiella pneumoniae Infection and NiO

Results of two replicate experiments, summarized in Table 1, indicate that enhanced mortality was observed among hamsters infected with K. pneumoniae within 1 hr after the intratracheal instillation of 5 or 7.5 mg of NiO. However, increase in death rates was not seen in hamsters given 1 or 10 mg of NiO nor in those challenged with the infectious agent 6 or 24 hr after the intratracheal instillation of NiO.

Table 1

MORTALITY OF HAMSTERS AFTER EXPOSURE TO NiO
AND CHALLENGE WITH K. PNEUMONIAE

Interval between NiO and <u>K. pneumoniae</u> challenge, hr	NiO, mg					
	<u>J*</u> <u>D/T**</u>	<u>%</u>	<u>1</u> <u>D/T</u>	<u>%</u>	<u>5</u> <u>D/T</u>	<u>7.5</u> <u>D/T</u>
1	0/12	0	0/12	0	5/12	42
6	4/12	33	0/12	0	3/12	25
24	0/12	0	0/12	0	0/12	0
NiO Control			0/12	0	0/12	0
					0/3	0
					0/6	0
					0/6	17
					0/6	17
					0/6	0

* 0.5% gelatin-saline.

** D/T = Dead/Total

2. Influenza Infection and NiO

a. Mortality

Replicate studies for the two virus-NiO sequences are summarized in Table 2. The results suggest enhancement of mortalities, especially upon exposure of infected hamsters to NiO. Death did not occur among 143 hamsters challenged with the influenza virus only, nor in 89 hamsters after intratracheal injection of 0.5% gelatin-saline per se. Furthermore, only 3 out of 266 hamsters died after intratracheal injection of NiO only. However, when infected hamsters were exposed to 2.5 and 5 mg of NiO within 24 and 48 hr after the infectious challenge, increased mortalities were observed. Because of the high death rates observed in control hamsters challenged with influenza virus and exposed to gelatin-saline, the statistical significance ($P < 5\%$) of the increase could be confirmed only for the group of hamsters exposed to 5 mg of NiO, 48 hr after the infectious challenge.

Intratracheal injection of NiO before the infectious challenge also appeared to enhance mortality rates; however, the increases were relatively small. In general, the death rates observed in this exposure sequence were markedly lower than those seen in hamsters exposed to NiO after the infectious challenge.

b. Histopathology

At 6, 24, and 48 hr after intranasal instillation of $1/5$ LD₅₀ of A/PR/8 influenza virus, groups of hamsters were injected intratracheally with 1, 5, and 7.5 mg of NiO, or gelatin-saline. All dead and moribund hamsters were autopsied and tissues were examined. The surviving hamsters were sacrificed at 2, 4, and 10 weeks and the tissues were obtained for

Table 2
MORTALITY OF HAMSTERS CHALLENGED WITH INFLUENZA VIRUS
AND EXPOSED TO NiO

Interval Between Virus and NiO hr	NiO, mg					
	0*		1		2.5	
	D/T	%	D/T	%	D/T	%
<u>Virus → NiO</u>						
1	0/30	0	1/30	3	0/30	0
24	30/48	63	26/47	55	24/30	80
48	4/25	16	2/25	8	4/25	16
<u>NiO Control</u>	0/45	0	2/42	5	1/45	2
<u>NiO → Virus</u>						
1	0/30	0	0/29	0	0/29	3
24	1/29	3	2/30	7	3/30	10
48	0/30	0	1/26	4	3/30	10
<u>NiO Control</u>	0/44	0	0/44	0	0/45	0

* 0.5% gelatin-saline

examination. Controls represented by hamsters given 1, 5, or 7.5 mg of NiO, gelatin-saline, saline, or the virus only were sacrificed at 2, 4, and 10 weeks after the respective treatments. All lung tissues were subjected to histopathological examination.

Control Hamsters. After administration of saline and gelatin-saline the pathological findings consisted of mild congestion with a mild interstitial response of a chronic nature (Figures 1-4). Some alveolar hemorrhage and atelectasis were observed, but these findings were attributed to the method of sacrifice of the animals. Hamsters challenged with the virus only, among which no deaths occurred, showed a mild chronic interstitial response with some congestion. The response to instillation of 1, 5, and 7.5 mg NiO was of an acute nature and consisted of an infiltration of polymorphonuclear cells and lung macrophages. The extent of the pathology appeared to be related to the amount of NiO administered (Figures 5-7). In lungs of hamsters receiving 7.5 mg of NiO, considerable consolidation, some alveolar hemorrhage, mild congestion, and marginal emphysema were observed.

Experimental Hamsters. Most of the hamsters challenged with the virus and given NiO, gelatin-saline, and saline, respectively, were dead one week after the infectious challenge. At necropsy, hamsters given 1, 5, and 7.5 mg of NiO at 6, 24, and 48 hr after the infectious challenge, showed a mild to severe acute interstitial response. The response consisted of interstitial infiltration of heterophils and macrophages, with the more severe response observed in the 7.5 mg NiO group. Lungs of hamsters challenged with the virus and exposed to saline or gelatin-saline and sacrificed at the same time periods (6, 24 and 48 hr post-challenge) did not show this type of interstitial reaction. The pathology was comparable to that found in hamsters given gelatin-saline or saline without the infectious agent.

Figure 1

VIRUS - GELATIN SALINE: Gelatin saline was administered 48 hr after challenge with virus and the hamster was examined 1 week post virus infection. The lung showed an acute interstitial reaction with mild congestion, and alveolar hemorrhage. Note the "thickened" bronchial epithelium.
95x

Figure 2

VIRUS - GELATIN SALINE: The gelatin saline was administered 48 hr after challenge with the virus and the hamster was examined 1 week after the virus infection. The pleural cells are "rounded up". No evidence of an exudate or fibrin deposition was present.
150x

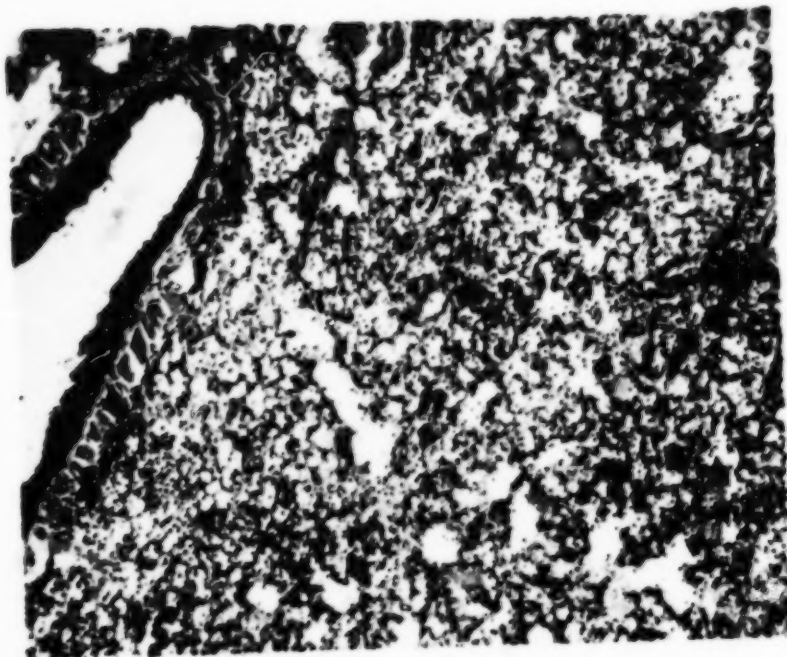


Figure 1

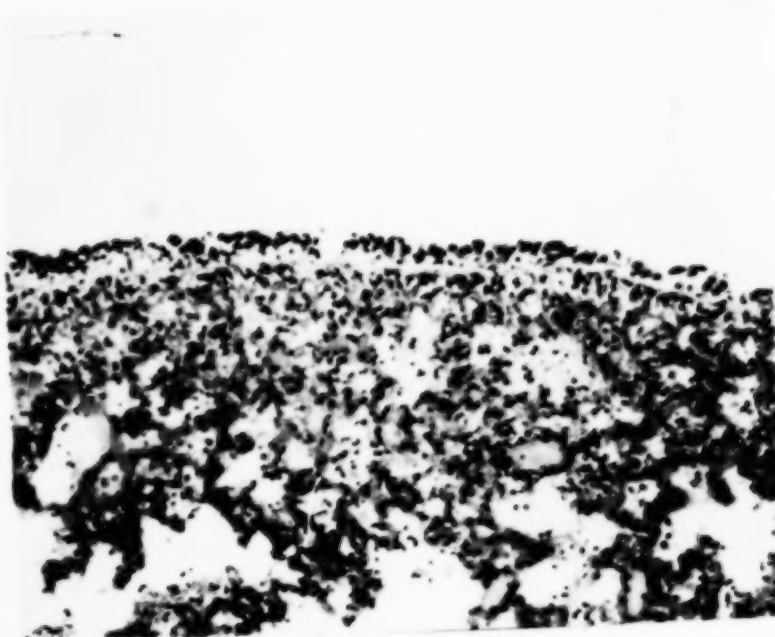


Figure 2

Figure 3

VIRUS - GELATIN SALINE: The gelatin saline was administered 24 hr after the challenge with the virus and the hamster was examined after 1 week. Beginning adenomatosis was observed surrounding some bronchioles. Note the "thickened" epithelium.
95x

Figure 4

VIRUS - SALINE: The saline was administered 24 hr after the challenge with the virus and the hamster was examined 4 weeks later. Extensive adenomatosis was present. Note the "thickened" epithelium.
95x

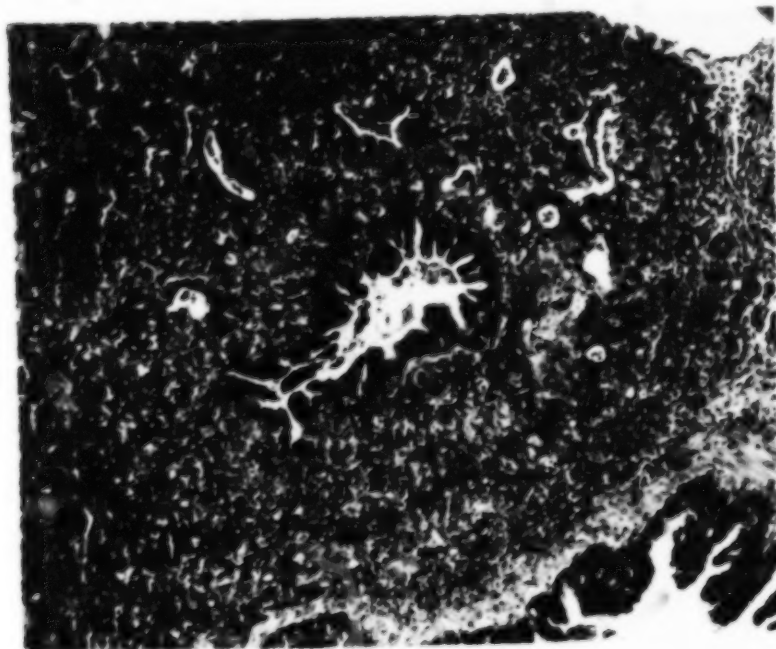


Figure 3

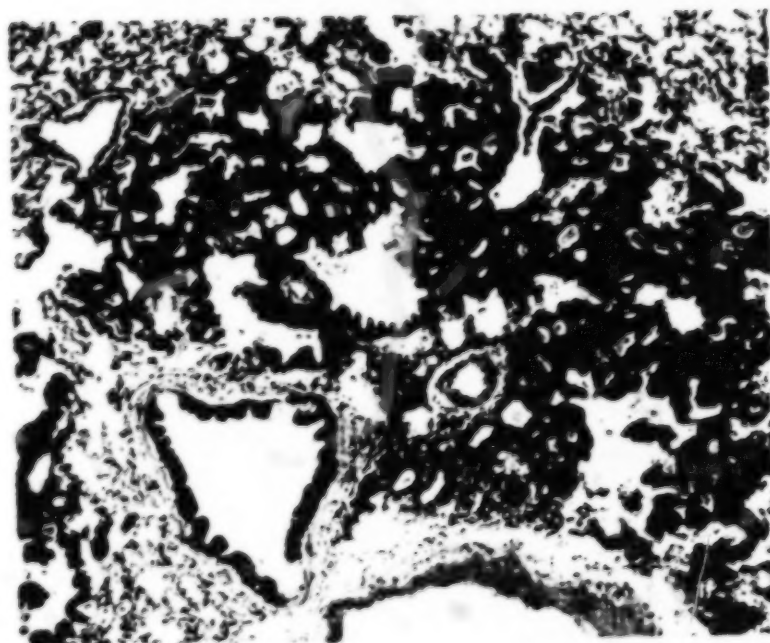


Figure 4

Figure 5

1 mg NiO IN GELATIN SALINE: The hamster was examined 2 weeks after NiO administration. The NiO particles were sparse with an accompanying mild interstitial reaction, mild atelectasis and some alveolar hemorrhage. Note the normal respiratory epithelium.
95x

Figure 6

5 mg NiO IN GELATIN SALINE: The hamster was examined 2 weeks after NiO administration. The NiO particles were distributed mainly in the hilus and generally surrounded bronchioles. Note the normal respiratory epithelium.
95x

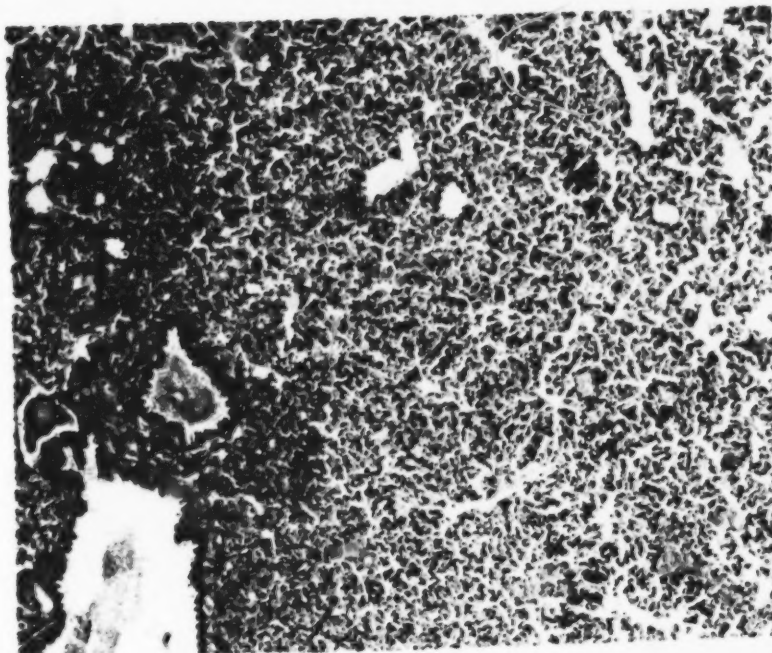


Figure 5

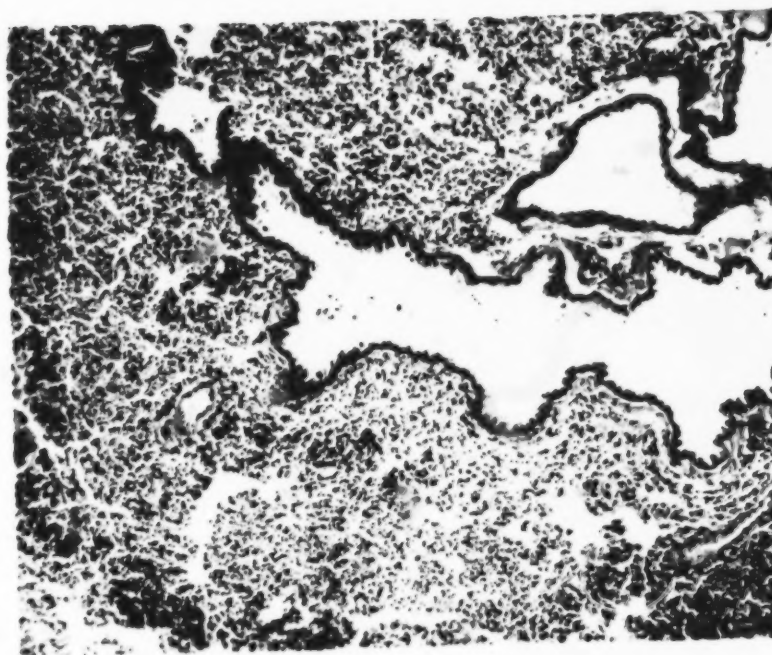


Figure 6

Figure 7

7.5 mg NiO IN GELATIN SALINE: The hamster was examined 2 weeks after NiO administration. The NiO is heavily concentrated in the parenchyma with an accompanying mild to moderate interstitial reaction. Note the normal bronchial epithelium.
95x

Figure 8

VIRUS - 1 mg NiO IN GELATIN SALINE: The NiO was administered 24 hr after the virus and the hamster was examined 2 weeks later. The pleura has greatly "thickened" with no exudate or other evidence of inflammation.
150x

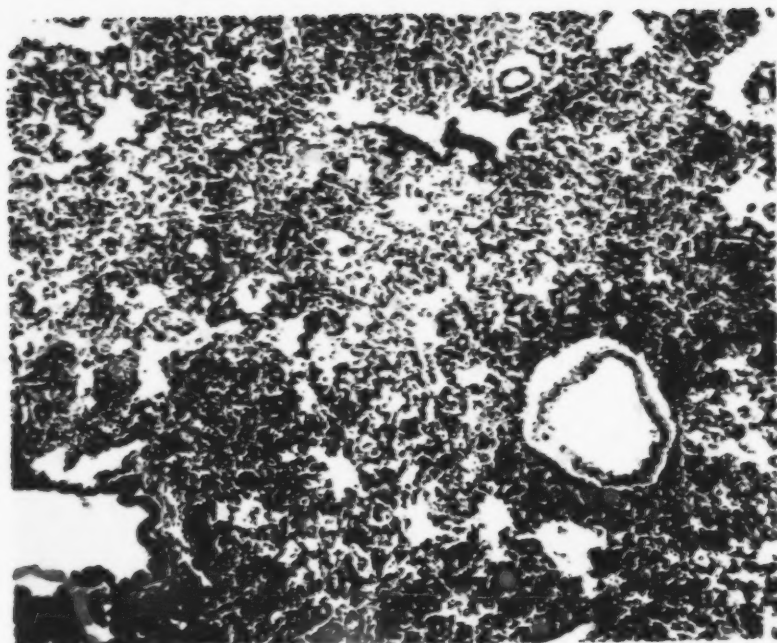


Figure 7

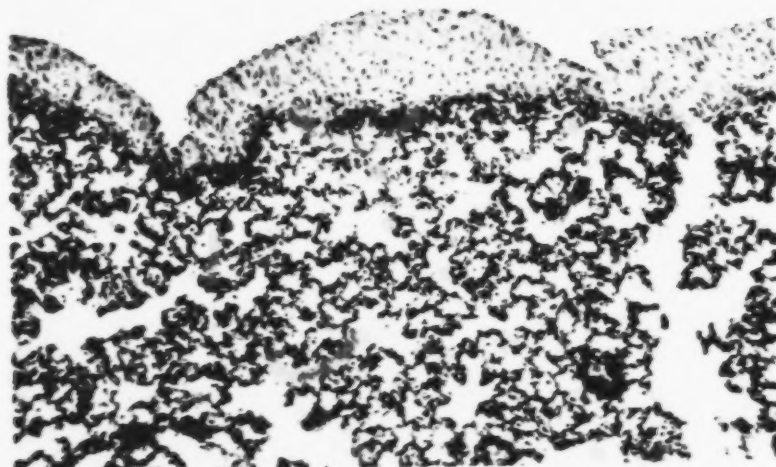


Figure 8

In addition to the NiO response all three groups of hamsters showed a markedly thickened bronchiolar and bronchial epithelium, similar to those seen in Figures 1, 3, and 4. In some instances, epithelial hyperplasia was present as evidenced by the number of mitotic figures visible in the basilar cells of the epithelium. In other instances, the epithelium appeared hyperplastic, without the presence of mitotic figures. Thus, the term "thickened" was applied when mitosis could not be demonstrated. Additionally, the pleural mesothelial cells in these hamsters appeared to "round up", (Fig. 2) and an occasional area of adenomatosis in the vicinity of a bronchiole was observed.

Hamsters examined at the 2 week interval included the various controls and those given 1, 5, 7.5 mg of NiO 6 hr after infectious challenge. The pathological changes in the lungs were similar to those seen in the 1 week group of hamsters, and consisted of interstitial response to the NiO, bronchial epithelial hyperplasia, and a pleural reaction which appeared as a thickened pleural lining (Fig. 8). Adenomatosis was more prominent at this time period.

A chronic interstitial reaction, with many macrophages containing NiO particles, was observed in lungs of hamsters examined 4 weeks after challenge with the virus. This response varied with the amount of NiO given as well as with the time period elapsed after the infectious challenge. The thickened bronchial epithelium was present in all groups. The adenomatosis (Fig. 4) was considerably more prominent in the 24 and 48 hr groups than in the 6 hr group. The pleural thickening was especially prominent in the 24 and 48 hr groups, in some cases reaching a depth of almost 1 mm.

Hamsters from all three time groups as well as control animals, killed 10 weeks after administration of virus, showed lung tissue changes similar to those found in those hamsters sacrificed 4 weeks after virus infection.

In general, an acute reaction related to the dose of NiO was observed upon histopathological examination of lung tissues. Furthermore, there appeared to be an additive effect of virus and NiO, virus and gelatin-saline or virus and saline. The effect appeared to be related to the time interval between infectious challenge and administration of NiO or gelatin-saline, as well as to the time elapsed after the challenge with the virus. Thickened or hyperplastic epithelium, adenomatosis, and thickened pleura were seen only in hamsters challenged with the virus in conjunction with NiO, gelatin-saline, or saline. In addition, there was a difference between hamsters exposed to the virus and NiO and those exposed to the virus and gelatin-saline or virus and saline, in that the NiO appeared to elicit an additional response above that produced by the gelatin-saline or saline.

c. Clearance of Virus from Lungs

To determine the effects of an intratracheal injection of NiO on the clearance rate of virus from lungs, groups of hamsters were challenged with influenza virus and 24 and 48 hr later were given 5 mg NiO, gelatin-saline, or saline. Hamsters, challenged with influenza virus only served as controls. Three hamsters from each group were sacrificed at 1 hr and at 1, 2, 3, 4, 5, and 6 days after the infectious challenge and the lungs were removed. The virus in the lungs was assayed from a 20% lung suspension titrated in eggs and measured by hemagglutination (HA) titers. The data, including the number of hamsters that died during the experiment, are shown in Table 3.

Table 3
CONCENTRATION OF INFLUENZA VIRUS IN LUNGS OF HAMSTERS
(Log₁₀/0.1 ml)

Time After Virus Challenge, Day	Virus Control	Interval Between Virus Challenge and Exposure					
		24 hr		48 hr			
		NIO	Sal	NIO	Sal	NIO	Sal
1 hr	2.5						
1	8.0						
2	6.5	8.0	6.5	8.0		7.5	7.39
3	6.5	7.45(3)*	7.33	7.0		4.0	6.66(1)
4	6.33	6.75(7)	5.55(5)	6.0(3)		5.5	5.33
5	4.66(1)	6.39(1)	5.75	4.5(2)		3.75	4.5
6	4.33	**	3.5	3.5		3.39	

* Number of dead hamsters in parenthesis.

** No surviving hamsters.

Among the hamsters challenged with the virus only, 1/23 died while in the groups given intratracheal injection of NiO 24 and 48 hrs after the infectious challenge, 11/22 and 4/16 died, respectively. In groups of hamsters given gelatin-saline 24 and 48 hr after the infectious challenge, 5/24 and 0/16 died respectively, and among those given saline 24 and 48 hrs after the challenge 5/21 and 1/16 died. Thus, in this experiment the mortality rate of hamsters challenged with virus and exposed to NiO was approximately twice that of the controls. In general, there was little difference in virus titers between the various experimental and control groups. However, it appeared that the concentration of virus was somewhat higher and the virus persisted for a longer time in lungs of hamsters exposed to NiO within 24 hr after the infectious challenge.

IV. Summary

Enhanced mortality was observed among hamsters infected with K. pneumoniae within 1 hr after intratracheal instillation of 5 and 7.5 mg of NiO. Similarly, increased mortalities were observed among hamsters challenged with influenza virus and exposed to NiO. However, because of the high death rate in control hamsters challenged with the virus and exposed to gelatin-saline, the statistical significance of the mortality increase could be demonstrated only for hamsters exposed to 5 mg of NiO within 48 hr after the infectious challenge.

Upon histopathologic examination of lung tissues, hyperplastic epithelium, adenomatosis, and thickened pleura were seen in hamsters challenged with the virus in conjunction with NiO, gelatin saline, or saline. In hamsters infected with the virus and exposed to NiO, the NiO appeared to elicit an additional response above that produced by gelatin-saline or saline.

Clearance studies of virus from lungs indicated little difference in virus titers between experimental and control groups of hamsters. Only in the group of hamsters exposed to NiO within 24 hr after virus challenge was there a somewhat higher concentration of virus which persisted for a longer period of time.

C. INTERACTION OF CHROMIUM TRIOXIDE AND RESPIRATORY
INFECTION IN MICE

I. Introduction

This phase of the studies was conducted to determine the effects of single and multiple exposures to chromium trioxide (Cr_2O_3) aerosol on the resistance of mice to influenza infection and bacterial pneumonia. In addition, limited studies were conducted to determine the effects of combined exposures to Cr_2O_3 aerosol and nitrogen dioxide (NO_2) on resistance to respiratory infections.

II. Materials and Methods

Experimental Animals. Specific pathogen free male Swiss-Webster mice, 5 to 6 weeks old, were used. Food and water were provided ad libitum.

Respiratory Agents. Influenza virus A/PR/8 was obtained from Dr. Max Rosenbaum, Naval Medical Research Unit No. 4, North Chicago, Illinois. This virus was passaged several times in mice and a 20% lung suspension was used for the infectious challenge. Prior to use in the studies, the virus was identified by use of specific A/PR/8 influenza virus antiserum obtained from the National Institutes of Health. Bacterial challenge was made with mouse adapted Klebsiella pneumoniae type A, strain A-D.

Administration of Cr_2O_3 . Mice were exposed for 2 hr inside a plexiglass chamber to Cr_2O_3 aerosol produced by the Wright Dust Feed Mechanism. The estimated concentration of Cr_2O_3 aerosol in the chamber ranged from 3 to 12 $\mu\text{g}/\text{liter}$ of air. Prior to dissemination, the Cr_2O_3 was passaged through a 100 mesh sieve to remove all particles larger than 20μ .

NO₂ Exposure. Mice were held in wire cages in a plexiglass chamber for the 2 hr exposure to 5 ppm of NO₂. The NO₂ concentration in the chamber was monitored at 30 min intervals using the Saltzman method (Anal. Chem. 26: 1949, 1954).

Histopathology. Upon autopsy, the mice were anesthetized with carbon dioxide (CO₂), the lungs and heart removed as a unit, and representative tissues were fixed in a 10% phosphate buffered formalin solution. After blocking in paraffin, 4 μ thick sections were cut in a rotary microtome, stained with hematoxylin and eosin, and examined.

III. Results and Discussion

1. Single Exposure to Cr₂O₃

In a preliminary study, mice were exposed to Cr₂O₃ aerosol for 1, 2, and 2-1/2 hr. Microscopic examination of lungs of the mice showed clumps of Cr₂O₃ particles distributed over the bronchial tree. Some particles were seen in the lower bronchioles with few particles found in alveolar macrophages. A slight acute interstitial response with some congestion was present.

In subsequent experiments mice were challenged with an estimated one LD₂₅ of airborne A/PR/8 influenza virus and at 1, 24, and 48 hr after the infectious challenge were exposed for 2 hr to Cr₂O₃ aerosol. The results of four replicate experiments summarized in Table 4 indicate that exposure to Cr₂O₃ aerosol had no effect on the susceptibility of mice to influenza infection. A second series of experiments were completed using the reverse sequence, i.e., groups of 20 mice were exposed to Cr₂O₃ aerosol for 1, 24, and 48 hr before the viral challenge.

The results of two replicate studies shown in Table 4 again indicate that inhalation of Cr_2O_3 did not enhance the susceptibility of mice to the influenza infection.

Table 4
MORTALITY OF MICE CHALLENGED WITH INFLUENZA VIRUS
AND EXPOSED TO Cr_2O_3 AEROSOL

<u>Interval Between Virus and Cr_2O_3, Hr</u>	<u>Mortality</u>	
	<u>D/T</u>	<u>%</u>
<u>Virus $\rightarrow$ Cr_2O_3</u>		
Virus Control	9/42	21
1	8/40	20
24	10/40	25
48	10/40	25
Cr_2O_3 Control	1/32	3
<u>$\text{Cr}_2\text{O}_3 \rightarrow$ Virus</u>		
Virus Control	30/80	38
1	10/40	25
24	9/40	22
48	17/40	43
Cr_2O_3 Control	0/8	0

2. Combined Exposures to NO₂ and Cr₂O₃

Two replicate experiments were carried out to determine the effects of combined exposures to NO₂ and Cr₂O₃ aerosol on the resistance of mice to influenza infection. The infectious challenge with the A/PR/8 influenza virus was followed 1 or 24 hr later by a 2 hr exposure to 5 ppm of NO₂ which was followed immediately by a 2 hr exposure to Cr₂O₃ aerosol. At 7 and 14 days after the infectious challenge the surviving mice were sacrificed and lung tissues were obtained for histopathological examination.

The results summarized in Table 5 suggest slight enhancement in mortality when mice were exposed to Cr₂O₃ aerosol or a combination of NO₂ and Cr₂O₃ within 24 hr after the infectious challenge. However, the differences in mortalities between the virus control and experimental groups of mice were not significant at $P \leq 0.05$. Mortalities did not occur among non-infected mice exposed to NO₂ or Cr₂O₃ aerosol or to the combination of the two pollutants.

3. Multiple Cr₂O₃ Exposures

To determine the effects of multiple exposures to Cr₂O₃, mice were exposed for 2 hr daily for 5 consecutive days to Cr₂O₃ aerosol. At the end of 1, 3, and 5 exposures, two mice were sacrificed and their lungs were subjected to histopathological examination.

After one exposure, large Cr₂O₃ particles were sparsely distributed in the alveoli and alveolar ducts and occasional particles were seen in the macrophages. Large numbers of particles were observed in the esophagus.

Table 5

MORTALITY OF MICE CHALLENGED WITH INFLUENZA VIRUS
AND EXPOSED FOR 2 HR TO NO_2 AND 2 HR TO Cr_2O_3 AEROSOL

<u>Treatment</u>	<u>Mortality</u>	
	<u>D/T</u>	<u>%</u>
<u>Controls</u>		
Virus	6/16	38
NO_2	0/8	0
Cr_2O_3	0/8	0
$\text{NO}_2 \rightarrow \text{Cr}_2\text{O}_3$	0/12	0
<u>1 hr*</u>		
Virus $\rightarrow \text{NO}_2$	6/12	50
Virus $\rightarrow \text{Cr}_2\text{O}_3$	4/12	33
Virus $\rightarrow \text{NO}_2 \rightarrow \text{Cr}_2\text{O}_3$	5/20	25
<u>24 hr*</u>		
Virus $\rightarrow \text{NO}_2$	3/9	33
Virus $\rightarrow \text{Cr}_2\text{O}_3$	5/10	50
Virus $\rightarrow \text{NO}_2 \rightarrow \text{Cr}_2\text{O}_3$	9/16	56

* Interval between infectious challenge and initiation of exposure to the pollutants.

After three daily exposures, a greater number of clumped large particles was seen in the main bronchioles. The number of smaller individual particles lining the alveoli and respiratory bronchioles as well as the number of phagocytized particles within the macrophages were greater than after the single exposure.

After five daily exposures to Cr_2O_3 aerosol even more clumped particles were present in the alveoli and respiratory bronchioles. Clumps of macrophages containing Cr_2O_3 particles were more prominent even at low magnification. A mild interstitial reaction to the particles was also observed.

Replicate studies were also conducted to determine the effects of repeated exposures to Cr_2O_3 aerosol on the mortality rate of mice challenged with K. pneumoniae and A/PR/8 influenza virus. Both experimental sequences, namely the infectious challenge followed by exposure to Cr_2O_3 aerosol and vice-versa, were included. Results of the experiments summarized in Table 6 indicate that repeated exposures to Cr_2O_3 aerosol did not enhance the mortality rates.

IV. Summary

Single or multiple exposures to Cr_2O_3 aerosol did not enhance mortality in mice challenged with K. pneumoniae or A/PR/8 influenza virus. Similarly, no significant increases in mortalities were observed among infected mice exposed to a combination of Cr_2O_3 aerosol and NO_2 . Histopathological examination of lung tissues indicated distribution of Cr_2O_3 particles in the alveoli and alveolar ducts and presence of phagocytized particles within macrophages. Thus, it would appear of interest to further determine the effects of exposure to Cr_2O_3 aerosol on phagocytic activity of macrophages and on the retention of infectious microorganisms in lungs.

Table 6

MORTALITY OF MICE CHALLENGED WITH INFECTIOUS AGENTS
AND REPEATEDLY EXPOSED TO Cr_2O_3 AEROSOL

Expt. Treatment	Mortality			
	Influenza Virus		K. pneumoniae	
	<u>D/T</u>	<u>%</u>	<u>D/T</u>	<u>%</u>
<u>A. Agent $\rightarrow$ Cr_2O_3*</u>				
Agent Control	12/20	60	31/60	52
Cr_2O_3 Control	0/10	0	0/10	0
Agent $\rightarrow$ Cr_2O_3	13/20	65	28/60	47
<u>B. $\text{Cr}_2\text{O}_3 \rightarrow$ Agent**</u>				
Agent Control	12/20	60	13/60	22
Cr_2O_3 Control	0/10	0	0/20	0
$\text{Cr}_2\text{O}_3 \rightarrow$ Agent	7/20	35	12/60	20

*Influenza virus challenge was 24 hr prior to first Cr_2O_3 exposure. K. pneumoniae challenge was 1 hr prior to first Cr_2O_3 exposure.

**All infectious challenges were 24 hr after final Cr_2O_3 exposure.

D. IMMUNE RESPONSE IN MICE DURING LONG-TERM NO₂ EXPOSURE

I. Introduction

Previous studies in our laboratories indicated that exposure of squirrel monkeys to low concentrations (1 to 5 ppm) of NO₂ affected the production of serum neutralization antibodies (1, 2). Therefore, a more extensive study was initiated to determine the immunological response in mice vaccinated with a highly purified influenza virus vaccine and exposed to various low concentrations of NO₂. Parameters of interest were hemagglutination-inhibition (HI) and serum neutralization (SN) antibody formation, mortality rate in mice challenged with live virus, lung histopathology, extent of lung edema, and serum immunoglobulin levels. In addition, scanning electron microscopic examination of lung tissue and macrophage were performed.

This long-term exposure study is continuing and only data accumulated to date are presented.

II. Materials and Methods

Experimental Animals. Specific pathogen free male Swiss-Webster mice 4 weeks old were obtained from Charles River Laboratories. After a two week quarantine period, the mice were placed in the respective environmental chambers and held for 2 days before initiation of the exposures. During the study mice were removed from the chambers for maintenance 3 times a week for 1 hr and clean cages were provided once a week. Throughout the experiments food and water were provided ad libitum.

Exposure to NO₂. To maintain control and experimental mice under similar conditions, three identical walk-in aluminum-lined chambers (4 x 6 x 6.5 ft) were used. Ambient air was passed through conventional fiber-glass filters before entering the exposure chambers. Air flow through the chambers was identical, and was at a rate of 20 changes/hour. The mean

temperature in the chambers was $75^{\circ} \pm 2^{\circ}\text{F}$.

Randomly distributed mice were housed in suspended wire cages, which were moved periodically to various positions on the cage racks. This permitted a thorough and unbiased exposure to the experimental environments.

For NO_2 exposures, a minute amount of NO_2 was continuously passed from a cylinder through a stainless steel tube into a glass mixing vessel where it was diluted and mixed with filtered air. The mixture was then passed into the NO_2 exposure chambers. To verify the homogeneity of NO_2 , air samples were taken from different sections of the chamber using a 100 ml flask as the sampler. The NO_2 concentration was determined and calculated by the Saltzman method (3) and in addition, a Mast gas analyzer was used for continuous monitoring of NO_2 .

In one chamber used for continuous exposure to NO_2 , the mean NO_2 concentration was 2.0 ± 0.3 ppm and ranged from 1.3 to 2.8 ppm over a 7 month period. In the second chamber in which mice were exposed to 0.5 ppm of NO_2 with daily 1 hr peaks of 2.0 ppm of NO_2 , the actual mean concentration was 0.56 ± 0.07 of NO_2 with a range of 0.4 to 0.9 ppm. The mean concentration for the 1 hr peaks was 1.9 ± 0.2 of NO_2 and ranged from 1.4 to 2.4 ppm over the 7 month exposure period.

Influenza Virus Aerosol Challenge. Mouse adapted influenza $\text{A}_2/\text{Taiwan}/1/64$ was obtained from Mr. Robert Bower, Abbott Laboratories, North Chicago, Illinois. This virus was passaged several times in mice and a 20% lung suspension was used in all challenge studies. Prior to use, the virus was identified by use of specific $\text{A}_2/\text{Taiwan}/1/64$ virus antiserum obtained from the National Institutes of Health.

Infectious aerosol challenge was conducted in a 350 liter plastic chamber (60 x 60 x 95 cm) installed within a microbiological safety cabinet. A University of Chicago Toxicity Laboratory type atomizer was used to produce airborne particles of 1 to 5 μ mass median diameter. The virus suspension was fed to the atomizer by a 50 ml syringe activated by a motor-driven piston delivering 0.4 ml/min. Filtered air was supplied to primary and secondary inlets of the atomizer at a flow rate of approximately 33 l/min. The humidity in the chamber was maintained at $78 \pm 6\%$ RH.

The aerosol was sampled with an all-glass impinger (AGI-30) employing PBS with 0.2% bovine serum albumin (BSA) as a collecting fluid. The inhaled dose of the virus was a function of the concentration of the virus in the air, respiratory minute volume of the mice, and the duration of exposure to the viral aerosol (4).

For infectious challenge, groups of vaccinated mice were placed in the aerosol chamber and exposed for 6 min to the mouse adapted influenza virus. After challenge, the mice were air washed for 10 min, removed from the aerosol chamber and held for 14 days in an isolated animal room in filter-capped cages. Non-vaccinated mice challenged with airborne influenza virus or 0.2% BSA served as controls.

Vaccine. Chicken embryo A₂/Taiwan/1/64 vaccine (Zonomune), lot No. BP0549, was supplied by Eli Lilly and Co., Indianapolis. Mice were given one subcutaneous injection of 279 CCA units in 0.1 ml vaccine.

HI and SN Titrations. Tests were performed in duplicate by the microtiter method (5) in disposable "v" plates (Cooke Engineering Co., Alexandria, Va.) as described by Davenport and Minuse (6). In all tests, 1% chicken red blood cells were used.

For the hemagglutination-inhibition (HI) test, four hemagglutinating units of antigen were used, all antisera were heated at 56°C for 30 min and were treated with trypsin-periodate to remove nonspecific inhibitors of hemagglutination.

The protocol used for serum-virus neutralization (SN) test was similar to that described in the USPHS Requirements (7). Sera were heat inactivated at 56°C for 30 min, serially diluted, and incubated for 1 hr at 4°C with an equal volume of influenza virus. The serum-virus mixture was then tested in 10 day old embryonated chicken eggs. The embryonated eggs were inoculated by the allantoic route with 0.1 ml of the virus-serum mixture, incubated at 37°C and harvested when an EID₅₀ dose of 32 to 320 was attained as indicated by hemagglutination of the virus control. The EID₅₀ was determined by parallel infectivity tests in eggs by using a 0.1 ml virus-saline mixture. As controls, phosphate-buffered saline (PBS), PBS plus normal mouse serum, and normal mouse serum plus virus were inoculated into the eggs.

Scoring of Pulmonary Lesions. The extent of pulmonary lesions was expressed as a percentage of the total lung consolidated (8). A score of 1 represented 25% lung consolidation, 2 = 50%, 3 = 75%, 4 = 100%, and a score of 5 represented death.

Lung Edema. To detect the extent of lung edema, three pools each consisting of 3 to 5 lungs for each experimental group were weighed immediately after removal from the mice. The lungs were then held in a vacuum desiccator and reweighed at 24 hr intervals until no additional weight loss was apparent.

The ratio of the wet-dry weights was used to express the extent of edema.

Lung Tissue - SEM. Lungs were fixed by airway perfusion with a paraformaldehyde-glutaraldehyde fixative (9). The mice were anesthetized and exsanguinated, and the lungs and trachea were removed and attached to a perfusion apparatus. Perfusion was continued for at least 2 hr with the lungs completely immersed in the fixative. Upon completion of airway perfusion, the trachea was ligated and the lungs were floated in fixative. The fixed tissue was washed in distilled water and dehydrated with increasing concentrations of ethyl alcohol. Thereafter, emyl acetate was substituted for the alcohol and the tissue was dried by the critical point method in carbon dioxide. The samples were mounted with an adhesive on copper specimen holders and to render their surface conductive they were covered by high vacuum evaporation with a thin layer of carbon followed by gold. The specimens were examined in a JEOL scanning electron microscope.

Alveolar Macrophages - SEM. Macrophages were obtained from mice through tracheobronchial lavage by the method developed for rabbits by Myrvik et al (10) and modified by Coffin et al (11).

Alveolar macrophages from combined lavage fluids of three mice were isolated from the surfactant by centrifuging at 300 xg for 10 min and subsequent repeated washing and centrifugation in saline. Total macrophage counts were made utilizing a white blood cell diluting pipette and counting the cells in a hemocytometer (11). Viability was determined by the dye exclusion technique (12).

The isolated macrophages were resuspended in PBS and placed in plastic Petri dishes containing a glass cover slip used as a substrate for the macrophages to attach. After 1 hr incubation in a constant temperature bath at 30°C the glass cover-slips were removed, rinsed with warm PBS and fixed in 1% glutaraldehyde solution. The fixed macrophages were washed with distilled water, immediately frozen in Freon-22 and freeze-dried in vacuum. The non-adherent macrophages remaining in the Petri dishes were centrifuged, washed in PBS and resuspended in 1% glutaraldehyde. After fixation the macrophages were washed in distilled water, smeared on coverslips, immediately frozen in liquid Freon, and freeze-dried.

For examination the coverslips were attached to metal specimen holders and the macrophages were covered with a thin layer of carbon followed by gold to render them conductive. Scanning electron micrographs were taken at 5 and 25 KV, respectively on a JEOL scanning electron microscope.

Experimental Protocol

Mice were exposed continuously to the following three environmental conditions:

- A. 2 ppm of NO₂
- B. 0.5 ppm of NO₂ with daily 1 hr peaks of 2 ppm of NO₂ for 5 days/week
- C. filtered air

After a 2 month exposure, the mice were vaccinated by a single subcutaneous injection of the influenza vaccine and were held in either NO₂ environment or filtered air. Groups of 14 to 20 mice were sacrificed at 2, 4, 8, and 12 weeks and the sera were pooled in groups of two. The sera were assayed for HI and SN antibodies, and portions of the sera were saved for detection of immunoglobulin levels. Additional bleedings are planned at 16, 20, 24, and 28 weeks of exposure.

To determine the protective effect of the vaccine as measured by mortality rates and lung lesion scores, groups of 20 mice were challenged with approximately one LD₅₀ of A₂/Taiwan influenza virus 4 weeks after the vaccination. Additional challenges will be made at 16 and 28 weeks post vaccination. Table 7 outlines in detail the protocol for the entire study.

III. Results

Body Weight. Mice from two randomly selected cages in each of the three chambers were weighed weekly. The initial mean body weights of 22 mice in 2.0 ppm NO₂ chamber, 21 in 0.5 ppm NO₂ chamber, and 30 mice in the control chamber were 30.0, 30.0, and 32.5, respectively. At the time of vaccination, 3 months after entering into the chambers, the mice exposed to NO₂ had gained 13 g while the control mice gained 9.5 g. At 9 weeks after vaccination, all three groups of mice weighed 44.5 g and at 16 weeks after the vaccination the mean weights were 44.2, 45.9 and 45.1 g, respectively. Thus, all groups of mice showed a consistent weight gain throughout the study, and all mice appeared healthy.

Serology. An HI antibody response was noted in all groups of mice 2 weeks after vaccination (Table 8). When the HI titers of mice exposed to filtered air were considered as control baseline value, no significant (4-fold) differences in HI titers were observed throughout the 12 week period.

The initial 2 week response as measured by SN antibody titers appeared to be depressed in mice exposed to NO₂ (Table 8). The mean SN antibody titer for the control group of mice was 1:34, whereas for mice exposed continuously to 0.5 ppm of NO₂ or those held prior to vaccination in filtered air and after vaccination at 2 or 0.5 ppm of NO₂, the titers ranged from <1:8 to 1:8. At the 4 and 8 weeks periods, however, the SN titers were comparable for all groups.

Table 7

PROTOCOL FOR IMMUNOLOGICAL STUDY IN MICE EXPOSED TO NITROGEN DIOXIDE

Exposure Group	Pre-vaccination Treatment for 3 Months	Vaccinate	Vaccination followed by Continuous NO ₂ Exposure and AB Titters at 2, 4, 8, 12, 16, 20, 24, 28 Weeks	Infectious Challenge for Mortality Study at 4, 16, 28 Weeks
A.	2.0 ppm (n = 600)	1. Initial Ab (n + 20)	(a) Ab titer on 20 mice given vaccine at each of above times. (n = 160)	(a) Challenge 20 vaccinated mice at each of the above times. (n = 60)
		+		
	NO ₂ exposure (n = 280)	(b) Saline (n = 60)	(b) Do not bleed saline mice.	(b) Challenge the saline mice as above. (n = 60)
	2. Initial Ab (n = 20)	(a) A2 Vaccine (n = 220)	(ab) Same as #1 above.	(ab) Same as #1 above.
		+		
	Filtered Air (n = 280)	(b) Saline (n + 60)		
B.	0.5 ppm with daily 1 hr peaks of 2.0 ppm (n = 600)	----- Same groups and exposures as above but with intermittent NO ₂ exposures -----		

Table 7 (Continued)

PROTOCOL FOR IMMUNOLOGICAL STUDY IN MICE EXPOSED TO NITROGEN DIOXIDE

Exposure Group	Pre-vaccination Treatment for 3 Months	Vaccinate	Vaccination followed by Filtered Air Exposure and AB Titters at 2, 4, 8, 12, 16, 20, 24, 28 Weeks	Infectious Challenge for Mortality Study at 4, 16, 28 Weeks
C. Controls (n = 900)	1. Initial Ab (n = 20)	(a) A2 Vaccine (n = 220)	(a) Ab titer on 20 mice given vaccine at each of above times. (n = 160)	(a) Challenge 20 vaccinated mice at each of the above times. (n = 60)
		(b) Saline (n = 60)	(b) Do not bleed saline mice.	(b) Challenge the saline mice as above. (n = 60)
	2. Initial Ab (n = 20)	(a) A2 Vaccine (n = 220)	(ab) Same as #1 above.	(ab) Same as #1 above.
		(b) Saline (n = 60)		
	3. Initial Ab (n = 20)	(a) A2 Vaccine (n = 220)	(ab) Same as #1 above.	(ab) Same as #1 above.
		(b) Saline (n = 60)		
	0.5 ppm NO ₂ with intermittent exposure (n = 280)	(a) A2 Vaccine (n = 220)	(ab) Same as #1 above.	(ab) Same as #1 above.
		(b) Saline (n = 60)		
	2 ppm NO ₂ (n = 280)	(a) A2 Vaccine (n = 220)	(ab) Same as #1 above.	(ab) Same as #1 above.
		(b) Saline (n = 60)		

Ab Studies: HAI and SN on pooled serum samples (pool consists of 7-10 groups of 2 mice)
Immunoglobulin assays on pooled serum samples (pool consists of 7-10 groups of 2 mice)

Total number of mice needed: 2100

Table 8
MEAN HI AND SN RECIPROCAL TITERS IN VACCINATED
MICE EXPOSED TO NO₂

NO ₂ , ppm		Reciprocal Titer at Weeks after Vaccination						
Pre- Vacc.	Post- Vacc.	HI ^a				SN		
		2	4	8	12	2 ^b	2 ^c	8 ^c
0	0	18	16	14	14	34	23	23
0.5	0.5	13	16	10	12	<8	31	29
2	2	17	11	7	7	12	46	26
0	0.5	33	16	11	30	8	26	19
0	2	12	8	8	14	8	26	36
0.5	0	9	19	16	14	10	15	21
2	0	6	23	12	15	20	40	21

^a Four antigen units of egg grown A₂/Taiwan virus were used in the HI test.

^b Tested against 258-320 EID₅₀ mice.

^c Tested against 21-32 EID₅₀ virus.

The rate of HI and SN seroconversion in vaccinated mice held in the various environments is shown in Table 9. The initial HI seroconversion rate was lowest in mice exposed to 2 and 0.5 ppm of NO₂ before vaccination and held in filtered air after the vaccination. The seroconversion rates were 29 and 20% respectively. Thereafter, only mice exposed to 2 ppm of NO₂ before and after vaccination, and those exposed to filtered air before and to 2 ppm of NO₂ after the vaccination showed consistently lower HI seroconversion rates.

The SN seroconversion rate presented an entirely different pattern. All mice in the control group had SN antibody within 2 weeks of vaccination, whereas none seroconverted among those continuously exposed to 0.5 ppm of NO₂ and the seroconversion rate for the remaining experimental groups ranged from only 40 to 57%. However, 4 weeks after vaccination, the seroconversion rate ranged from 80 to 100% in the various experimental groups. These data are in agreement with those previously reported from our laboratories, whereby squirrel monkeys injected with mouse-adapted influenza virus showed an initial delay in SN, but not in HI antibody response.

Infectious Challenge. Vaccinated mice and control mice injected with saline were challenged with airborne A₂/Taiwan influenza virus 4 weeks after vaccination. Mortality data shown in Table 10 indicate that the vaccine afforded satisfactory protection against challenge with the infectious virus in all mice. Because of the low challenge dose, which resulted in only 15% mortality in the control mice, the significance ($p \leq 0.05$) of the observed differences in protection between saline and vaccinated mice could be ascertained only for the following groups of mice:

Table 9
RATE OF SEROCONVERSION IN VACCINATED MICE*

NO ₂ , ppm		Weeks after Vaccination							
Pre- Vacc.	Post- Vacc.	HI Response > 8, %		SN Response > 8, %		SN Response > 8, %		SN Response > 8, %	
		2	4	8	12	2	4	8	
0	0	71	75	71	57	100	100	100	100
0.5	0.5	60	67	29	57	0	100	71	71
2	2	70	25	29	14	50	88	86	86
0	0.5	100	71	67	83	43	86	67	67
0	2	50	30	33	43	40	80	67	67
0.5	0	20	60	67	56	56	80	63	63
2	0	29	86	44	44	57	86	78	78

* Based on 14 to 20 mice per group.

Table 10

MORTALITY RATE AND LUNG LESIONS IN MICE CHALLENGED
WITH INFLUENZA VIRUS 4 WEEKS AFTER VACCINATION

NO ₂ , ppm		Mortality			Lung Lesion Score	
Pre-Vacc.	Post-Vacc.	Saline	Vaccine		Saline	Vaccine
		D/T	D/T	%		
0	0	3/20	0/20	15	1.95	0.30(*)
0.5	0.5	9/20	0/20	45(**)	3.20(**)	0.35(*)
2	2	1/19	0/20	5	2.37	0.35(*)
0	0.5	3/20	0/19	15	2.05	0.42(*)
0	2	7/20	0/18	35	3.00(**)	0.44(*)
0.5	0	9/20	2/20	45(**)	3.40(**)	1.15(*) (**)
2	0	7/20	3/19	35(**)	2.90	1.21(*) (**)

(*) Significant differences ($P \leq 0.05$) between vaccinated and corresponding saline-injected mice.

(**) Significant increase ($P \leq 0.05$) within vaccinated or saline control groups when compared to mice held in filtered air only.

- a. mice exposed to filtered air,
- b. mice continuously exposed to 0.5 ppm of NO_2 ,
- c. mice exposed to 0.5 ppm of NO_2 before vaccination and held in filtered air after vaccination,
- d. mice held in filtered air before vaccination and exposed to 2 ppm of NO_2 after vaccination.

When lung lesion scores were used as the indicator of influenza infection (Table 10) all vaccinated mice showed significantly lower scores than the corresponding control mice..

While the differences in mortality rate within the various vaccinated groups were not significant, lung lesions were found to be significantly more severe in mice exposed to 0.5 ppm or 2 ppm of NO_2 before vaccination and held in filtered air after the vaccination than in the other vaccinated groups. Thus, a 3 month exposure to NO_2 before vaccination exposure to filtered air appeared to reduce the resistance to infection of vaccinated mice as measured by lung lesions.

Several significant differences were observed when the mortality rates were compared within the various groups of mice given saline and exposed to the experimental conditions. The death rates among the mice exposed to 0.5 or 2 ppm of NO_2 before injection of saline and then maintained in filtered air were significantly higher ($p \leq 0.05$) than among those exposed continuously to 2 ppm of NO_2 or to filtered air. In addition, the mortality rates were significantly higher in groups of mice exposed continuously to 0.5 ppm of NO_2 after injection of saline than among those exposed continuously to 2 ppm of NO_2 .

Using lung lesion scores as another indicator of influenza infection, it was again noted that mice exposed to 0.5 ppm of NO₂ for 3 months before saline injection and then held in filtered air exhibited significantly ($p \leq 0.05$) higher lung lesion scores than control mice, mice exposed continuously to 2 ppm of NO₂, and those held in filtered air prior to saline injection and then exposed to 0.5 ppm of NO₂. Interestingly, mice exposed continuously to 0.5 ppm of NO₂ exhibited more severe lung lesions than control mice or mice exposed to filtered air prior to saline injection and then held at 0.5 ppm of NO₂. Therefore, it appeared that 3 months exposure to NO₂ before injection of vaccine or saline resulted in the development of more severe influenza lung lesions. In the saline groups a higher mortality rate was found in the same pre-exposure groups than in the control group.

Lung Edema. The wet:dry lung weight ratios are shown in Table 11. At 2, 4, and 8 weeks after vaccination, no significant differences were found between the filtered air control and the experimental values. However, 12 weeks after vaccination, i.e., approximately 6 months after initial NO₂ exposure the ratios were significantly higher in mice continuously exposed to 0.5 ppm of NO₂ than in the control mice. All other values were not significantly different from the controls.

SEM of Lung Tissue. Lung tissues from mice exposed to 2 ppm of NO₂ for 3 months appeared normal when examined by the SEM. The alveoli were cup shaped and not distended. Inter-alveolar septa were not broken and possessed small inter-alveolar pores (Fig. 9A and 9B). Tracheobronchial epithelium and lung tissues of mice exposed to 2 ppm of NO₂ for 4 months showed changes attributable to NO₂. The non-ciliated epithelial cells of the tracheobronchial tree showed loss of microvilli and the presence of small holes or pits (Fig. 9C). These cells were considered to be degenerating

Table 11
WET/DRY LUNG WEIGHT RATIO

NO ₂ , ppm		Post-Vaccination Means			
Pre-Vacc.	Post-Vacc.	2 wk	4 wk	8 wk	12 wk
0	0	4.53	4.68	4.64	4.11
0.5	0.5	4.54	4.50	4.55	4.79*
2.0	2.0	4.70	4.54	4.65	3.88
0	0.5	4.78	4.52	4.48	4.72
0	2.0	4.66	4.39	4.64	4.08
0.5	0 0	4.58	4.58	4.50	4.44
2.0	0	4.46	4.87	4.54	4.60

* Significant difference ($P \leq 0.05$) from other values at a given time period.

cells and were not seen in control animals exposed to filtered air (Fig. 9D). Furthermore, the lung tissues from mice exposed to NO₂ showed emphysematous changes which, although not numerous, could be readily recognized. The alveoli were enlarged and coalesced to form enlarged airspaces. The alveolar septa were lost and strands of tissue or trabeculae remained. Inter-alveolar pores, visible in some septa, were enlarged and irregularly shaped (Fig. 9E and 9F).

SEM of Macrophages. Macrophages isolated from vaccinated mice after 21 weeks of continuous exposure to 2 ppm of NO₂, 0.5 ppm of NO₂ with daily peaks of 2 ppm of NO₂, and to filtered air, were examined in the SEM. The macrophages were pooled from lung lavages obtained from 3 or 4 mice in each exposure group and the total counts and viability are shown in Table 12.

Figure 9

SCANNING ELECTRON MICROGRAPHS OF MOUSE LUNG TISSUE:
(A & B) Lung tissue from mice exposed to 2 ppm NO₂ for 3 months. (C & D) Non-ciliated epithelial cells of the tracheobronchial tree exposed to 2 ppm NO₂ for 4 months and the air control, respectively. (E & F) Lung tissue from mice exposed to 2 ppm NO₂ for 4 months.

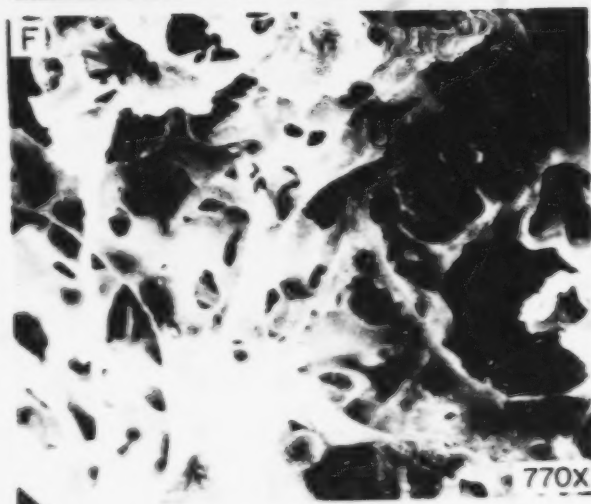
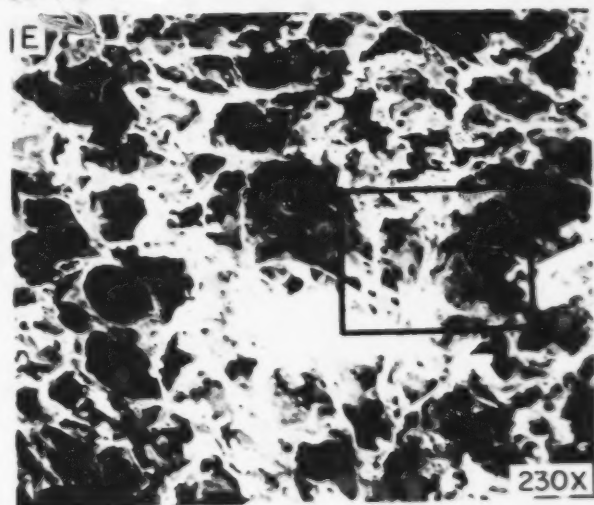
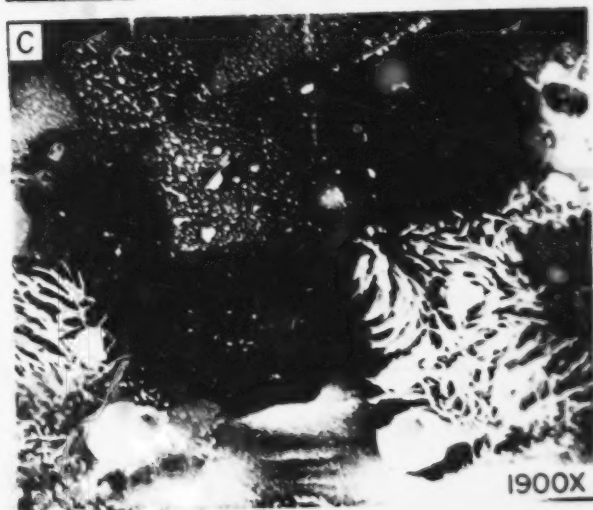
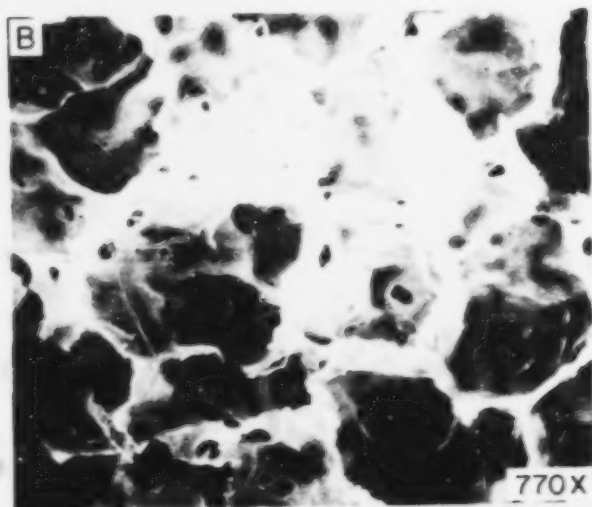
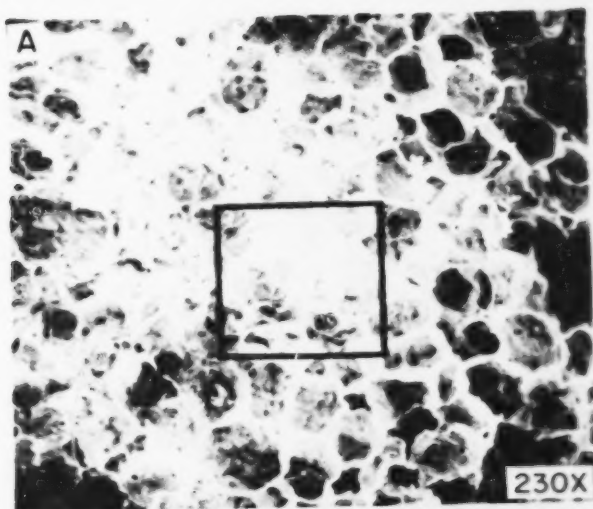


Figure 9

Table 12

TOTAL COUNTS AND VIABILITY OF
ALVEOLAR MACROPHAGES OBTAINED FROM MICE

<u>NO₂, ppm</u>	<u>Total Count x 10⁶</u>	<u>Viability %</u>
0	5.55	96.0
2	9.15	93.5
0.5	4.05	94.5

Scanning electron micrographs are shown in Fig. 10. Macrophages grown and fixed directly on cover slips are shown in Fig. 10A-10C. The intricate surface structure of a normal alveolar macrophage with the surface membrane extensions attaching to the glass substrate is seen in Fig. 10A. Macrophages obtained from mice after continuous exposure to 2 ppm of NO₂ for 21 weeks showed distinct alteration in the surface structure as seen in Fig. 10B. The changes became more extreme after 21 weeks of continuous exposure to 0.5 ppm of NO₂ with daily peaks of 2 ppm of NO₂ (Fig. 10C), whereby a complete deterioration of the macrophages with only some skeletal remnants still attached to the glass was seen.

Macrophages not attached to the glass substrate but remaining in suspension were fixed and smeared onto cover slips. Normal macrophages shown in Fig. 10D showed the intricate structure with numerous surface projections. However, the attachment to the glass cannot be seen and the macrophage appeared to be somewhat more rounded than the one shown in Fig. 10A. Unattached macrophages obtained from the 2 ppm of NO₂ exposure group (Fig. 10E), were more severely damaged than those grown on the cover glass (Fig. 10B). An almost complete

Figure 10

SCANNING ELECTRON MICROGRAPHS OF MOUSE PULMONARY ALVEOLAR MACROPHAGE CELLS: (A, B & C) grown onto glass substrate and fixed in situ; (D, E & F) recovered from the cell suspension after removal of the glass substrate and fixed in suspension. (A & D) Normal controls, (B & E) Macrophages obtained from animals after 21 weeks of continuous exposure to 2 ppm NO₂, and (C & F) after 21 weeks of continuous exposure to 0.5 ppm NO₂ with a superimposed 1 hr long daily peak of 2 ppm NO₂.

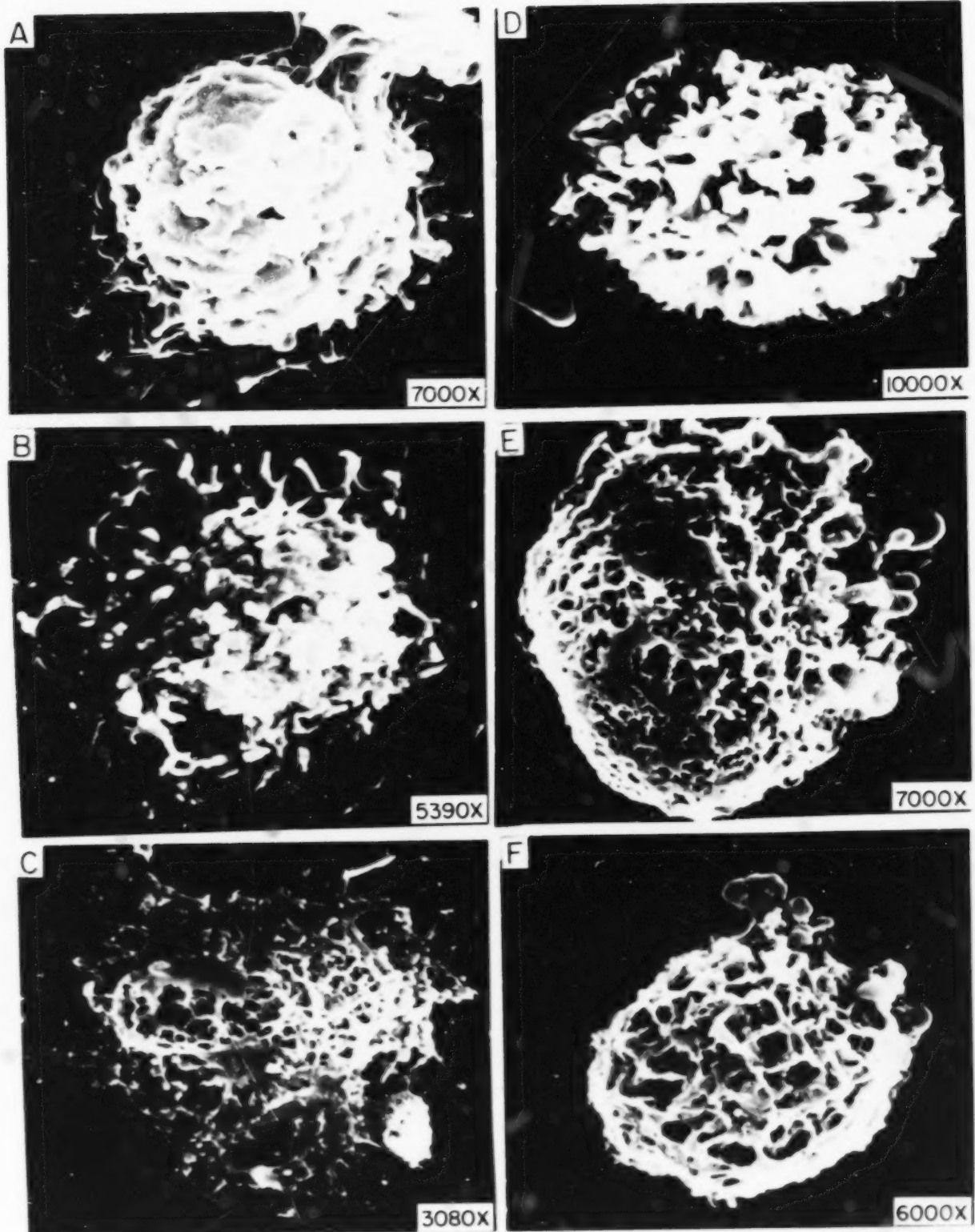


Figure 10

absence of surface processes and the presence of numerous fenestrae characterized the morphological condition of these cells. The distinct difference between the appearance of these cells and those spontaneously grown on cover slips may reflect the ability of the macrophages to attach to glass and their state of morphological deterioration. It is interesting to make the same comparison for macrophages from the 0.5 ppm of NO_2 exposure group. The unattached macrophage shown in Fig. 10F, while quite similar to the unattached cell from the 2 ppm of NO_2 exposure group, is different from the macrophages isolated from mice exposed to 0.5 ppm of NO_2 that did attach to the glass substrate (Fig. 10C). The macrophages attached to the glass appeared in a more advanced state of deterioration than those found in the maintenance medium.

IV. Summary

A study was initiated to determine the effects of continuous long-term exposure to NO_2 on the immune response in mice. After 3 months exposure to NO_2 or to filtered air, mice were injected with A_2 /Taiwan influenza virus vaccine or saline and placed in various experimental environments. The NO_2 exposure was either to 2 ppm or to 0.5 ppm of NO_2 with daily 1 hr peaks of 2 ppm for 5 days a week. The initial SN antibody response was depressed in mice exposed continuously to 0.5 ppm of NO_2 or those held in filtered air prior to vaccination and exposed to 2 ppm or 0.5 ppm of NO_2 after vaccination. The SN seroconversion rate was 100% in the control mice 2 weeks after vaccination, whereas no mice exposed to 0.5 ppm of NO_2 seroconverted. In addition, the rate for the remaining experimental groups ranged from only 40 to 57%. No significant differences in HI titers were noted throughout the study.

After challenge with live A₂/Taiwan influenza virus, mortality rates and lung lesions scores indicated protection in all vaccinated mice. In both vaccinated mice as well as those given saline, more severe influenza lung lesions were found in those animals held in NO₂ for 3 months prior to vaccination than in the filtered air controls.

The wet:dry weight lung ratios were examined at 2, 4, 8, and 12 weeks after vaccination. Lung edema was found only in mice held for 6 months (12 weeks post vaccination. Lung edema was found only in mice held for 6 months (12 weeks post vaccination) in 0.5 ppm of NO₂.

Scanning electron microscopic examination of lung tissues indicated that mice exposed to 2 ppm of NO₂ for 4 months showed changes attributable to NO₂. The non-ciliated epithelial cells of the tracheobronchial tree showed loss of microvilli and emphysematous changes appeared in the lung tissues.

Scanning electron microscopic examination was made of alveolar macrophages from vaccinated mice exposed to either 2 ppm or 0.5 ppm of NO₂ for 21 weeks. Macrophages from both groups showed distinct morphologic alterations when compared to the filtered air controls. In some cases there was complete deterioration of the cells with only some skeletal remnants.

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